Is daily replication necessary when sampling cortisol concentrations in association studies of children with autism spectrum disorder? A systematic review and discussion paper

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Abstract: Salivary cortisol may be used as a biomarker of stress and anxiety in children with an autism spectrum disorder (ASD). Some suggestions have been made that the measurement of cortisol needs to be undertaken by repeated days' observations to ensure reliability of the data obtained. These requirements are discussed in regard to 14 studies of the test-retest agreement and stability in cortisol data across repeated daily measurements. Results of those studies almost universally fail to support the argument for repeated daily measurements of cortisol. Implications for the research protocols of studies using cortisol as an index of stress in children with ASD are discussed.

Keywords: autism; cortisol; daily rhythm; stress.

Introduction

Stress in autism spectrum disorder

Autism spectrum disorder (ASD) is a neurological disorder that is also often comorbid with elevated stress (Corbett et al., 2009), anxiety (van Steensel et al., 2011), and depression (Bitsika and Sharpley, 2015) during childhood and adolescence. The prevalence of stress, anxiety, and depression in these children is several times that reported for children without ASD (White et al., 2009) and constitutes a significant confound for their interaction with their peers and achievement in school settings (Kim et al., 2000). Due to the reported difficulties in social communication that characterise ASD (APA, 2013), the precise degree of stress experienced by these children may be difficult to determine, and so attention has been given to biological indicators of that stress, most usually by using cortisol as an indicator of chronic hypothalamus-pituitary-adrenal (HPA) axis activation, which accompanies ongoing stress (Dallman, 1993; Young et al., 2008). A cascade of HPA responses commences in the hypothalamus and moves to the pituitary gland and adrenal cortex, from where cortisol is released into the bloodstream about 8 min after the onset of the stressor (Guyton and Hall, 2006) and into saliva about 10 min later (Buono et al., 1986).

Cortisol as an index of stress

These responses are initiated by two distinct processes. The first of these processes is referred to as the diurnal rhythm (DR) and describes variations in HPA axis activation that are initiated by the circadian clock within the hypothalamus. The DR exhibits a maximum concentration of cortisol about 45 min after waking in the morning and a nadir during the early evening (Clow et al., 2010). This process conducts cortisol to body tissues where it binds to glucocorticoid receptor proteins present on the surface membranes of most cells and enters the cell nucleus acting as a transcriptional regulator. Depending on the specific tissue and gene, the glucocorticoid response may be inhibitory or stimulatory (Aron et al., 2007). Cortisol affects intermediary metabolism; calcium homeostasis; the immune system; other endocrines; skin and connective tissue; breast, lung, and cardiovascular systems; and mood, appetite, sleep, memory, and vision (Guyton and Hall, 2006). The second process for releasing cortisol is via non-DR immediate HPA axis responses to stressors within

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the environment (Sapolsky et al., 2000). This response is the origin of the 'stress hormone' title given to cortisol and assists the organism's survival by increasing heart rate and blood flow, stimulating gluconeogenesis in the liver to ensure adequate glucose for heightened brain activity (Guyton and Hall, 2006), and inhibiting several pro-inflammatory cytokines (such as IL-1β, TNFα, IL-6) and promoting anti-inflammatory cytokines (such as IL-10). This discussion will focus upon the first of these responses – i.e. the DR and its reliable measurement in children with ASD.

The HPA axis diurnal rhythm in ASD

Chrousos (2009) commented that malfunction of HPA axis responses in the form of DR dysregulation 'might impair growth, development, behaviour and metabolism, which might potentially lead to various acute and chronic disorders' (p. 380), including anxiety and depression (Schiefelbein and Susman, 2006). Thus, measurement of DR dysregulation in children with ASD can provide an insight into their overall neurobiological and psychological states that can be used to identify those children who are 'at-risk' of developing anxiety and depression, as well as those children whose HPA axis dysregulation may be a potential source of other physiological anomalies that could adversely influence their overall health.

There have been some comments that there is variability in the DR among children with ASD (Taylor and Corbett, 2014). These comments have been followed by suggestions that the reliable measurement of the DR in these children therefore requires repeated sampling over several consecutive days. Although that recommendation has not been made either firmly or specifically in the literature about ASD and has not been validated by any data regarding a precise index of the assumed variability in these children, it has achieved some standing in the field, with some evidence of the adoption of a repeated-sampling protocol in some studies (e.g. Corbett et al., 2008; Kidd et al., 2012), even though that repeated sampling has not produced any clear indication of its necessity (i.e. by demonstrating that the DR variability was either sufficiently severe as to invalidate single measures or that multiple day's observations overcame that variability). Clearly, the presence of significant between-days DR variability would argue for such a repeated-sampling protocol. However, in the absence of any conclusive data to support that hypothesised variability, the imposition of a multiple-days data collection protocol upon children who already find social interactions challenging (APA, 2013) could constitute a stressful experience in itself, thus unintentionally increasing HPA axis DR variability and providing a confound to the data collected.

Generalisability of cortisol DR data

The issue here is one of the generalisability of the HPA axis DR data obtained from 1 day to other days. This is a methodological issue that is concerned with producing data from a sample of the DR (i.e. over a day) so that those data are reasonably consistent with the data that might be obtained from the total population of samples (i.e. over all days). There is a literature on this issue of the number of sampling repetitions needed to obtain salivary cortisol data that are generalisable to the population of all such samples, and a review of that literature can inform this discussion.

Methods

Studies reviewed

A search of PubMed and Google Scholar was undertaken in May 2016, using the criterion 'salivary cortisol daily variation/rhythm reliability,' for papers that were reported in English in a peer-reviewed journal and were focussed upon the issue of the generalisability of data from 1 day to other days. Although the initial search with no time limit and the descriptors listed above produced over 10 000 references (suggesting that the two search engines covered the field adequately), these papers were then reduced in number by focussing the search upon papers published since 2000, as the use of saliva to assay cortisol is a relatively recent laboratory practice. In addition, papers were excluded if they did not report data relevant to the issue of the number of observations needed for reliable measurement of the DR. Hand-searching was also used to identify any other papers from the reference lists of relevant papers. Most papers reported associations between salivary cortisol and other variables, and relatively few were focussed upon the issue of the number of observations needed for reliable measurement of the DR. Only 14 studies were identified that met these criteria and were relevant to the discussion here. To clarify the overall findings from these studies, each is described briefly below and in Table 1, with the final column in Table 1 presenting author quotations from those papers about the conclusions they reached.

Table 1: Studies of the variability in cortisol concentrations over time.

Authors	Participants	Sampling times	Sampling days	Time between samples	Cortisol measures	Results	Conclusions
Wust et al. (2000)	509 adults	Waking, 30, 45, and 60 min later	2 days	Consecutive days	0, 30, 45, 60 min Area under the curve (AUC) Mean increase 0–60 min after waking	Correlations between days 1 and 2: Waking r=0.37 30 min r=0.51 45 min r=0.62 60 min r=0.66 Mean increase r=0.47 AUC r=0.63	'Intraindividual stability over time was remarkably high' (p. 79)
Edwards et al. (2001)	42 adults	Waking, 15, 30, 45, 180, 360, 540, 720 min later	2 days	Consecutive days	Waking response (0–45 min) Diurnal pattern (0–180, 360, 540, and 720 min later)	Waking response r=0.52 Diurnal pattern r=0.65	'All measures showed reasonable stability across two sampling days and reliable indices of trait characteristic' (p. 1093)
Hucklebridge et al. (2005)	24 adults	Waking, 15, 30, 45, 180, 360, 540, 720 min later	2 days	Consecutive days	Waking response (mean of 0, 15, 30, 45 min) Daily response (0, 180, 360, 540, 720 min)	Waking response r=0.76 Daily response r=0.57	'Remarkably stable across 2 days' (p. 54)
O'Connor et al. (2005)	74 10-year-old	Waking, 30 min later, 4 pm, 9 pm	3 days	Consecutive days	Waking 30 min 4 pm 9 pm	Internal consistency: Waking=0.49 30 min later=0.77 4 pm=0.58 9 pm=0.75	'Moderate' stability (p. 213)
Rotenberg et al. (2012)	232 children 9–18 years	Waking, 30, 45 min later, before lunch, before dinner, bedtime	3 days	Variable, 1–64 days	Aggregate and single-time measures	ICC for single measures: Waking=0.40 Before lunch=0.37 Before dinner=0.41 Bedtime=0.21	'Moderate stability' (p. 1987)
Shirtcliffe et al. (2012)	357 children 9–15 years	Waking, afternoon (3:00–7:00 pm), just before going to bed	3 days	Consecutive days	Growth curves of total variance in cortisol to detect trait-like stability	Stability of cortisol across sampling times=34.9% of variance	'Substantial epoch-specific stability' (p. 497)
Platje et al. (2013)	229 boys, 181 girls 15 years	Waking, 30, 60 min later	3 days	Each day 1 year apart	Wake up level 30 min later 60 min later AUC	Rank order stability coefficients: Waking=0.15 30 min=0.24 60 min=0.24 AUC=0.29 (all p<0.001)	'Moderate to low rank order stability…due to stable trait factor and variable state factors' (p. 277)

Table 1 (continued)

Authors	Participants	Sampling times	Sampling days	Time between samples	Cortisol measures	Results	Conclusions
Segerstrom et al. (2014)	(1) 124 younger adults	Waking, 0.5 h later, 12:00 pm, 5:00 pm, 9:00 pm.	3 days	(1) Day 1, Day 44, Day 101, Day 137, Day 166	Diurnal slope Diurnal mean AUC	Reliability: 0.10 0.24 0.19	'The proportions of variance in diurnal cortisol parameters are similar to… anxiety and anger' (p. 307)
	(2) 148 older adults	Waking, 5:00 pm, 9:00 pm	2 days	(2) 6 months	Diurnal slope Diurnal mean AUC	0.10 0.27 0.11	
Ross et al. (2014)	(1) 130 children and adolescents	1, 4, 9, 11 h after waking	10 days	6 months	Total daily output and diurnal slope	ICC[a]: Daily output ICC=0.54 Diurnal slope=0.25	'Modest stability between visits', but, due to high variability, 'focussing on short-term cortisol fluctuations… (would be) fruitful' (p. 190) because they reflect the adaptive, dynamic activity of HPA axis (p. 191)
	(2) 147 Female adolescents	Waking, 0.5, 1, 4, 9, 14 h later	6 days	1 year	CAR, diurnal slope, total daily output	CAR=0.11 Diurnal slope=0.23	
	(3) 47 adults	Waking, 0.5, 1, 4, 9, 14 h later	12 days	2–4 months	CAR, diurnal slope, total daily output	Total daily output=0.48 CAR=0.22 Diurnal slope=0.47 Total daily output=0.55	
Wang et al. (2014)	580 adults, two studies (MESA1, MESA2)	MESA1: waking, 30 min later, 10:00 am, 12:00 pm, 6:00 pm, before bed MESA2: waking, 30 min later, 1 h after breakfast, 10:00 am, 12:00 pm, 4:00 pm, 6:00 pm before bed	3 days	Approx. 6 years	Wake up cortisol CAR (AUC)	ICC of means over 6 years: Wake up cortisol=0.11 CAR=0.09	'Significant changes in cortisol features over long periods', leading to difficulties in identifying a stable trait (p. 319)
			2 days	Approx. 6 years	Early slope (30 min–2 h after waking) Late slope (2 h after waking–bedtime)	Early slope=0.25 Late slope=0.42 AUC=0.05	
Laurent et al. (2014)	96 children 3–6-year old	30 min after waking, 30 min before bedtime	2 consecutive days	Monthly for 2 years, 32 months no collection, then 6-monthly for 2 years	Lagged effect over days sampled	Significant ($p<0.001$)	'Sample-wide tendency for stability in HPA activity over time' (p. 141)
Hankin et al. (2015)	224 youth (9–15 years)	Stress reactivity tasks	Pre- and post-stressors	1.5 years	AUC for baseline vs. post-stressor	$r=0.41$ over 1.5 years	'Significant test-retest stability' (p. 54)
Doane et al. (2015)	82 adolescents ($M=18$ years)	Waking, 30 180, 480 min later, bedtime	3 consecutive days	5, 9 months after first assessment	Mean values at time of observation	Latent trait factor for cortisol	'Highly stable across months' (p. 21)
Sharpley et al. (2015)	16 boys ASD (8–14 years)	30–45 min after waking	2 days	8.3 months apart	Mean values at time of observation	$\rho=0.575$, $p=0.020$	'Large… strong association' over time (p. 210)

[a]Intra-class correlation.

Results

Data from studies

Table 1 presents the 14 studies, almost all of which showed significant agreement in cortisol concentrations across days. Wust et al. (2000) reported a range of significant correlations between 0.37 (waking cortisol) to 0.66 (60 min after waking) and various other measures of cortisol concentrations from 509 adults without ASD over 2 consecutive days, observing high stability in those concentrations, commenting that the 'stability over time was remarkably high' (p. 79). Edwards et al. (2001) similarly found correlations of 0.52–0.65 for waking cortisol and the DR from samples collected on 2 consecutive days from 42 adult participants without ASD, noting the presence of 'reasonable stability' (p. 1093) in these values. Hucklebridge et al. (2005) reported a correlation of 0.76 in waking cortisol and 0.57 for the DR across 2 days from 24 adults without ASD and commented that 'there is little intra-individual variation (in cortisol concentrations) from 1 day to the next' (p. 52). O'Connor et al. (2005) collected cortisol samples from 74 10-year-old children over 3 consecutive days and found that data were 'remarkably stable' (p. 54), ranging from r=0.49 to r=0.77 in those concentrations across those days. Rotenberg et al. (2012) reported 'moderate stability' (p. 1987) with an average correlation of 0.41 between late afternoon concentrations of salivary cortisol samples collected from 233 children without ASD over 3 days, similar to that collected at waking (0.40). Shirtcliffe et al. (2012) examined cortisol concentrations from 357 children without ASD collected on 4 days over 6 years, reporting 'a very high degree of trait-like stability' over this period (p. 498). Platje et al. (2013) measured cortisol concentrations after waking in 410 adolescents on 1 day in each of 3 consecutive years and found that there were increases in mean level of cortisol concentrations over the 3 years, which were attributed to the effects of development during adolescence (i.e. not due to variability in the DR *per se* but rather related to developmental changes in participants). They also found that there was only low to moderate stability in the rank-order of participants' cortisol concentrations over the 3 years (0.15–0.29) although these values were statistically significant (p<0.001). Platje et al. (2013) argued that these relatively low stability values were a function of HPA axis activity being partly due to stable 'trait' (i.e. genetically determined) factors plus variable 'state' factors (i.e. participants' responses to daily events). Segerstrom et al. (2014) collected salivary cortisol from 124 college students (non-ASD five times/day for 3 consecutive days on five different occasions and from 148 older adults three times/day for 2 days, 6 months apart). The pooled results indicated that approximately 10% of the variance in cortisol concentrations was attributable to stable differences between participants and that this degree of person variance was 'similar to several dimensions of daily mood such as anxiety and anger' (p. 307). Segerstrom et al. (2014) also conducted a decision study of the number of days' measures needed to reach a test-retest reliability of 0.6 and concluded that, for diurnal slope (i.e. the DR), cortisol samples would need to be collected in 15 days. Although mathematically responsible, these suggestions do not acknowledge the difficulty in obtaining repeated samples of saliva from some children with ASD nor the stressful effect that such repeated measurements of cortisol for over 2 weeks might have upon them, thus potentially confounding the outcomes of tests for associations between stress, anxiety, and cortisol concentrations. [As shown in a later study described below by Sharpley et al. (2014), reaching a test-retest agreement of about 0.6 was achieved with only 2 single days' observations several months apart, this providing some challenge to Segerstrom et al.'s estimations.] Ross et al. (2014) collapsed data from three studies, two of which were on healthy adolescents without ASD and one on healthy adults without ASD. Data on cortisol concentrations were collected four or five times/day between 6 and 12 days and indicated that 50% of the variance in cortisol concentration total daily output, plus the cortisol awakening response (CAR) and DR, was attributable to day-to-day fluctuations, which they described as representing a 'state' for HPA axis activity. Those authors commented that the daily variation in cortisol concentrations was a function of health and social interactions and that, to achieve a stability of 0.6 over days of sampling of total daily output of cortisol, salivary cortisol would need to be collected at five periods that were 3 months apart and each of which consisted of 3 days' sampling. To achieve the same level of stability for the CAR would require 35 visits, each of which included 2 days of sampling. These finding also represent a logistic challenge for researchers working with children with ASD. However, adding a caveat to the recommendation for collecting salivary cortisol samples on multiple days, Ross et al. (2014) commented that cortisol's state-like pattern of variability was indicative of covariation with psychosocial and health variables and self-reports of physical well-being. Those authors stated that, due to this linking of cortisol concentrations with (variable) psychological and physical health indices, 'stability estimates are not likely to be substantially improved by simply increasing the number of consecutive sampling days' (p. 191) because the psychological and physical correlates of

cortisol concentrations will themselves not remain stable over those periods of sampling. Wang et al. (2014) found difficulty in identifying a stable 'trait' for cortisol concentrations in adult data collected 6 years after initial observation of multiple sampling of cortisol during a single day, but Laurent et al. (2014), who sampled nearly 100 children 3–6 years of age in 2 consecutive days each month for 2 years, then 6 monthly for 2 years, found significant stability in cortisol concentrations collected 30 min after waking and 30 min before bedtime. Hankin et al. (2015) collected cortisol from a sample of 224 children and adolescents (9–15 years) who underwent two laboratory stress reaction tasks 18 months apart and found 'significant test-retest stability' (p. 54) of 0.41 over this period. Both Doane et al. (2015) and Sharpley et al. (2015) collected data from adolescents or children on 3 and 2 consecutive days, respectively about 8 months apart and found highly stable associations between those measures. These data support the presence of stability in cortisol concentrations over quite long periods of time, with only one study failing to report data from test-retest observations that was not significantly correlated.

Trait and state aspects of HPA axis DR

Although only investigated by relatively few of these studies, the model of combined trait and state factor influence upon HPA axis activity that was referred to by Platje et al. (2013) and Ross et al. (2014) had been suggested by Adam et al. (2006) several years previously in their study of 158 older adults whose salivary cortisol was collected at waking, 30 min later, and at bedtime on 3 consecutive days, plus self-reports on 22 emotional states (e.g. loneliness, sadness, anger, threat, lack of control, etc.) at bedtime. In that study, higher levels of physical symptoms and fatigue were inversely associated with waking cortisol; higher levels of tension and anger were associated with flatter DR slopes; and higher levels of feeling lonely, sad or overwhelmed were associated with higher waking response cortisol concentrations (measured at waking and 30 min later). Lag effects were also noted in the significant correlations between emotional state on 1 day and cortisol concentrations on the next day, suggestive of possible causal links between emotional state and cortisol concentrations. These findings are of interest because they begin to define the ways in which different emotional states contribute to different aspects of the cortisol response and support the notion of a trait and state model of HPA axis activity.

Is 'stability' of the DR meaningful or possible?

The data reported in Table 1 therefore present a complex picture of the ways in which the cortisol DR might be assessed. On one hand, there are repeated reports of significant agreement between cortisol concentrations over quite long periods of time. Conversely, there is evidence that cortisol concentrations will vary according to physical and psychological health, which is an accepted source of error in any study of physiological or psychological variables. The breakdown of cortisol concentrations into trait and state components may be of value in understanding how the variability in DR that is referred to by some authors comes about. That is, almost any organism will demonstrate state variability over time, and it is unlikely (as noted by Ross et al., 2014) that repeating measurements over a series of days will produce cortisol data that do not show the (natural) variability attributable to changes in physical and/or psychological health. In fact, that kind of variability in physical and psychological status is commonly the target of researchers who test for agreement between (say) anxiety and cortisol concentrations in children with ASD (e.g. Bitsika et al., 2015). While it is almost never suggested that repeated measures of the psychological or physical health variables need to be undertaken (simply because that kind of variability represents the usual human state), it might also be the case that cortisol should be regarded as innately variable because of its association with those other health state variables. That suggestion has been confirmed by the finding of variability in repeated daily data on anxiety and mood that were collected from children (Cousins et al., 2011) and adolescents (Fuligni and Hardway, 2006). Assuming the presence of a relationship between cortisol and these mood states (as has been demonstrated), it may be concluded that the variability in cortisol could be a function of variability in mood states rather than 'instability' of the HPA axis itself. Under that model, variability in both physiological (cortisol) and psychological (anxiety, depression) states would be accepted as a normal condition of life and not requiring repeated measurement in cross-sectional studies of the association between HPA axis DR and mood or physical variables.

What to 'generalise'?

Instead, the problem of generalisability of the HPA axis DR data becomes one of sample size, where any measure is a sample from a universe of possible observations and

the measure obtained is a combination of the 'true' score plus measurement error (Webb et al., 2007). The closer the sample size is to the population, the greater is the reliability or generalisability of the sample taken. In this case, the variability in cortisol DR represents normal variability across observations due to psychological and/or physiological states (which might be called measurement error in this case), and the generalisability of a specific observation depends upon the relationship between the sample and the population. If the population chosen is the total range of cortisol DR measurements possible for a given individual, then the sample would have to approximate that population (i.e. repeated observations of the same individual's DR). However, if the population chosen is the HPA axis DR data from the population of all (say) boys aged between 6 and 12 years with ASD, then the sample's generalisability to the population would depend upon the relationship between the sample (i.e. the number of ASD boys) and that population (i.e. all ASD boys), both in a numerical sense (i.e. the sample of ASD boys would represent a large enough proportion of the population to allow for generalisation of the results collected from the sample to those assumed to be present in the population) and in a comparability sense (i.e. that the sample is not distinguishably different to the population on some important indices, such as IQ, age, ASD characteristics, etc.). If this interpretation of generalisability is accepted, then the issue of reliability of the cortisol DR becomes one of sample size of the group of participants (rather than their HPA axis DR) and their similarity (their age, IQ, etc.) to the population of all ASD boys. Thus, researchers might assume that their sample does represent the population in enough key aspects (e.g. for studies of children with ASD, these might include gender, age, IQ, ability) and is large enough to allow for some degree of generalisability of the sample data to the population.

As mentioned above, most of the studies shown in Table 1 were not conducted on children with ASD and therefore leave open the possibility that the data reproduced there do not apply to those children. However, one study from Table 1 did include children with ASD (Sharpley et al., 2015), providing some substance to the applicability of those data in the table to other children with ASD. Furthermore, there are data concerning the comparability of repeated observations of DR across ASD and children without ASD, although those data were not subject to the kind of test-retest evaluation reported in the studies in Table 1. That is, Corbett et al. (2008) presented 6 days of data from morning, afternoon, and evening cortisol sampling of 22 children with ASD and 22 children without ASD. Those authors commented that 'both the neurotypical children and the children with autism shown expected normal peak-to-trough rhythms' (p. 232).

Discussion

Summary and implications for studies of cortisol in children with ASD

Several points arise from this discussion for consideration in the planning of studies that collect DR salivary cortisol in samples of children with ASD. Although it is sometimes suggested, the need for repeatedly measuring DR profiles over several days is significantly challenged by the data regarding the presence of acceptable stability in cortisol concentrations from the majority of studies described in Table 1, plus the comment by Ross et al. (2014) that simply increasing the number of consecutive sampling days of data collection will not ensure stability. The presence of the trait versus state components of HPA axis activity, which may influence the DR of various individuals, is dependent upon their genetic makeup as well as the environmental stressors they encounter. That is, some individuals may be genetically inclined towards greater or lesser DR variability, as is indicated by studies of the serotonin transporter 5-HTTLPR (Karg et al., 2011; Sharpley et al., 2014) and glucocorticoid receptor (DeRijk et al., 2008), both of which are up-regulated as a result of stress and adversity during infancy and childhood (Weaver et al., 2004) and each of which influences HPA axis responsivity. As well as a considerable literature that reports increased anxiety among children with ASD (for reviews, see Kim et al., 2000; van Steensel et al., 2011), there is evidence that these children experience interpersonal peer-related stress in the form of bullying (van Roekel et al., 2010; Cappadocia et al., 2012), which is a major stressor in their lives and might be expected to contribute to up-regulation of these and other genes that influence the HPA axis.

Overall, good science is minimalistic in that possible confounds are reduced as far as is congruent with accurate measurement. As has been known for some time, any repeated measures design is vulnerable to the confounding effects of time such as history, maturation, and testing as well as possible intrusion from instrumentation effects, selection, and mortality when participants with ASD are recruited for studies that involve repeated collection of saliva (Cook and Campbell, 1979).

Logistic concerns

There is a further logistic issue that needs to be considered in this discussion, that is, the feasibility of obtaining repeated measures of HPA axis DR over several days. If, for example, Ross et al.'s (2014) suggestion of 3 days' sampling over five periods each 3 months apart for the DR, or 35 visits of 2 days each for the CAR, were followed, then it is highly unlikely that research on HPA axis responses could be obtained from children with ASD (or any other children) simply due to the invasive natures of such a research protocol. This is particularly relevant when collecting data from children with ASD who may find the repeated imposition even more disturbing than their peers without ASD. Parents of these children may (would!) refuse to participate if they believed that their children (or themselves) might be subject to overly demanding procedures such as those involved in multiple measures, thus limiting the possible participant sample to those parents and their children who may be most resistant to the stressful effects of such procedures and biasing any sample that does agree to these procedures. These factors become exaggerated when the repetition of sampling is extended over time, as would be the case were the findings regarding the required number of samples needed to meet the reliability levels described above by Segerstrom et al. (2014) and Ross et al. (2014) implemented in studies using children with ASD. Those suggestions, while mathematically rigorous for the population without ASD, do not represent practical methodologies when researching children with ASD.

Conclusion

The presence of measurement error in the form of variability within the DR across days may represent actual changes in HPA axis responsivity to physiological and/or psychological stimuli that are the common experience of all organisms. Attempts to reduce that variability may be attempts to reduce the research setting to an unrealistic extent, thus also reducing the generalisability of such repeated observation data to actual life.

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